Micro- and Nanotechnologies for Intracellular Delivery

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Review

Micro- and nanotechnologies for intracellular delivery

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Abstract

Majorities of drugs and biomolecules need to be delivered into cells to be effective. However, the cell membranes, a biological barrier, strictly resist drugs or biomolecules entering cells, resulting significantly reduced intracellular delivery efficiency. To overcome the cell membranes, a variety of intracellular delivery approaches including chemical and physical ways has been developed in recent years. In this review, we will focus on summarizing the nanomaterial routes involving in making use of a collection of receptors for targeted delivery of drugs and biomolecules and the physical ways of applying micro- and nanotechnologies for high-throughput intracellular delivery.

1. Introduction

Majorities of therapeutic targets are located within cell cytoplasm or nucleus. Thus drugs are only effective when they enter cells. However, the cell membranes, one of the most common biological barriers serving to isolate, protect and regulate cell from external environment, strongly limit the transport of drug molecules into the cytoplasm and nucleus and result in poor therapeutic efficacy. To overcome the barrier of the cell membranes and increase intracellular

delivery efficiency, many chemical and physical approaches involving micro- and nanotechnologies have been developed in the last decades. Chemical approaches are focused on applying nanostructured materials, made from polymers, lipids, peptides, inorganic and metallic substances, for intracellular delivery of a wide range of drugs, genes or other biomolecules.[1-6] In these systems, to achieve specific and targeted delivery, the surface of nanomaterials are often modified with certain targeting ligands, including antibodies, peptides, small molecules and so on, which have strong affinity with the receptors of targeted cells. Different from chemical approaches, physical methods usually simply disrupt the cell membranes to increase their permeability, thus facilitating gene or drug intracellular transport. Modern physical tools can be tailored to precisely deliver molecules into specific areas of cells with nano-size resolution, or designed to be suitable for high-throughput intracellular delivery. Herein, we will review a variety of micro- and nanotechnologies with focus on receptor mediated intracellular delivery and advanced physical platforms for transporting materials to cells.

2. Receptor-mediated endocytosis of nanomaterials for drug delivery

Nanomaterials have been playing important roles in biology and medicine, offering a wide variety of new strategies for biomedical applications including drug delivery[7,8] and gene therapy.[9-11]With nanomedicines, drug molecules can be better delivered to tumors in cancer therapy due to passive targeting phenomenon, also known as the enhanced permeation and retention (EPR) effect. To further improve the targeted delivery efficiency and extend the application scopes to other diseases, active targeting is generally employed as well by attaching specific ligands (e.g., antibodies, peptides and small molecules) to nanomaterials to recognize and selectively bind to specific cell receptors which are overexpressed on certain cell surface such as tumor cells.[12] For these reasons, design of new nano-platforms is of great significance

to advance targeted drug-delivery systems and cancer theranosis. To improve the design, it is imperative to firstly understand how targeted delivery is achieved through the aid of receptors.

2.1. Pathways of receptor-mediated endocytosis

The concept of receptor-mediated endocytosis was first put forward by Goldstein and Brown in 1974. It was observed that the binding of the low density lipoproteins (LDL) to human fibroblasts exhibits two different affinities, which was explained due to the specific receptor on fibroblast cell surface.[13] Nowadays, receptor-mediated endocytosis has been well recognized as a main route through which animal cells internalize ligands and macromolecules.[14,15] Endocytosis occurs constitutively in all mammalian cells and plays fundamental roles such as nutrient uptake, iron transportation and intracellular communication. Typically, nanomaterials transport through the cell membrane via four endocytic pathways including phagocytosis, macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis (**Figure 1**).[15,16] Among these pathways, clathrin-mediated endocytosis via specific receptor-ligand association is the best described mechanism. Previously, "receptor-mediated endocytosis" was referred to this pathway only. However, recently, an alternative non-specific endocytosis via clathrin-coated pits was also demonstrated.[17]

Clathrin-mediated endocytosis initiates when ligands bind to receptors on the cell surface and then the receptor/ligand complexes slide laterally into clathrin-coated or smooth pits. The coated pits are uniquely featured by protein clathrin. In opposite to this, smooth pits are lack of clathrin coating. Next, the pits bud into the cell and form primary endosomes where the ligand and receptor can separate. After internalization, the endosomal compartments continuously acidify from the original neutral pH (~7) on cell surface to pH (~4) in lysosomes. After membrane shaping into early endosomes, several primary endosomes fuse to form larger vesicles while the clathrin coating is shed. These larger vesicles are characterized by a unique morphology with a main vesicular and multiple tubular compartments. Usually, but not in all

cases, the detached ligands are carried to lysosomes for degradation, while the receptors cycle back to the cell surface to selectively recognize and bind new ligands.[18-21]

2.2. Receptors for targeted delivery of nanomedicines

A rapidly growing tumor requires a large amount of vitamins, essential trace elements and nutrients. Thus, cancer cells overexpress a wide range of tumor-specific receptors, which can be applied as targets to particularly deliver therapeutic drugs and imaging agents into tumors.[22] Currently, more than 25 specific targeting ligands have been noticed to involve in receptor-mediated endocytosis.[23] For example, antibodies and antibody fragments,[24-26] nucleic acid aptamers,[27-29] peptides,[30, 31] vitamins,[32,33] and glycoprotein[34, 35] have been considered as tumor-specific moieties to construct "guided molecular missiles".[36] These targeting receptors serve two purposes. One is to increase the likelihood that nanomaterials find their way into the tumor mass via both passive and active targeting and remain within the tumor. The other is to trigger the transport of the nanomaterials across the cellular membranes in pathological tissue. Next, we will focus on reviewing various types of cell receptors which can be harnessed for efficient targeted cancer therapy and early diagnosis.

2.2.1. Antibodies and antibody fragments

Owing to the high specificity and strong affinity to tumor receptors, mono-antibodies (mAbs) and their fragments are the most widely used targeting molecules. So far, about 30 of them have been approved for clinical use. A conventional method of incorporating mAbs and their fragments to targeting drug delivery systems is to conjugate to nanomaterial surface directly or through linker molecules.[37] Commonly, the conjugated manner of the antibody to nanomaterials is random without specific sites, which can be carried out by using carbodiimide-mediated chemistry. This approach is achieved by creating a stable amide bonds between amine groups in the antibody or antibody fragment and the carboxylic acid groups in nanocarriers.[38]

Alternatively, another approach is site-specific, realized with maleimide chemistry. Such binding strategy involves native or pre-designed thiol-containing cysteine residues that locate away from the antigen binding sites to assure full activity of mAbs and their fragments.[39] In this part, several most commonly utilized antibody receptors will be stated.

Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a type of single-chain trans-membrane protein receptor overexpressed in most epithelial cancer cells and fulfills crucial physiological roles including binding epidermal growth factor (EGF) and activating multiple signaling pathways and intracellular communications.[40] The EGF-EGFR interaction is among the first studied growth factor ligand-receptors.[41] Through the signaling pathway, EGF-EGFR can have a great influence in regulating cell proliferation, differentiation, and survival.[42,43] In recent years, due to the overexpression of EGFR found in various types of cancer cells, many approaches have been developed for designing receptor-mediated targeted drug delivery systems for cancer detection and treatment.[44] Nie *et al.* put forward an EGFR targeted multifunctional nanoparticles for drug delivery and in vivo dual-model imaging. As shown in **Figure 2a**, schematic illustration of quantum dots (QDs) functionalized with an amphiphilic polymer and then conjugated with ScFvEGFR targeting molecules. Figure 2b reveals the highly selective internalization between cancer cells presenting high level of EGFR (MDA-MB-231 cell) and low level of EGFR (MCF-7 cell). Although both cell lines were incubated with ScFvEGFR-QDs, obviously strong red fluorescence appears in EGFR over-expressed MDA-MB-231 cells while only very weak red signals are in EGFR down-expressed MCF-7 cells. Likewise to the MCF-7 group, a very low level of red fluorescence was observed in the group of cells with high EGFR expression but incubated with QDs possessing no ScFvEGFR modification. These results are convincing evidences that ScFvEGFR-conjugated nanoparticles have good potential in tumor imaging application.[45]

Vascular endothelial growth factor receptor (VEGFR)

Both tumor hypoxia and angiogenesis can upregulate expression levels of vascular endothelial growth factor (VEGF) in tumor cells, thus resulting in a corresponding overexpression of vascular endothelial growth factor receptor (VEGFR) on tumor endothelial cells. The VEGFR is regarded as the most relevant persuader in tumor angiogenesis.[46] There are two kinds of endothelium-specific receptor associated with angiogenic actions: VEGFR-1 and VEGFR-2. VEGFR-1 is responsible for physiologic process, in particular the angiogenesis development, and its function may be affected by many factors, such as different developmental stages, tumor cell types and various physiologic and pathologic conditions; VEGFR-2 is critical to mediate mitosis, angiogenesis, and permeability-enhancement and simultaneously plays a pivotal role in tumor progression.[47] Zhang and co-workers demonstrated that nanostructured lipid carriers (NLC) loaded with chemotherapeutic drug docetaxel and fluorescein FITC exhibit much superior antitumor cytotoxicity against B16 cancer cells if the nanomaterials are modified with anti-VEGFR-2 antibody.[48] Figure 2c shows the schematic representation of the NLC carriers and their fluorescence microscopy images. A relatively stronger green fluorescence is observed in cells incubated with antibody modified NLC (tNLC) whereas much lower fluorescence intensity can be detected in cells treated with non-targeted NLC (nNLC). In vivo anti-tumor experiments verified again the high targeting ability of the modified nanocarriers, as exhibited in Figure 2d, thus rendering the tNLC with the best efficiency of cancer treatment.

Prostate-specific membrane antigen (PSMA)

Prostate-specific membrane antigen (PSMA) is a kind of type II transmembrane glycoprotein produced by prostatic epithelium and with 100 kDa molecular weight and 750 amino acids. The expression of PMSA is mostly upregulated in prostate cancers and participates in membrane recycling, tumor metastasis and tumor aggressiveness. However, the expression of this antigen

has also been found in normal and extraprostatic tissues, such as small bowel, lymph node and bone metastases.[49,50] Due to the highest PSMA expression related to high-grade tumor, PSMA could be clinically used as a specific ligand for targeting to metastatic and malignant tumors in vivo.[51] Anti-PSMA antibodies modified nanomaterials are the most wildly developed PSMA-involved approach for targeted therapeutics and diagnostics. Up to now, many antibodies have been approved for clinical use, such as J59, 7E11-C5 and MDX-070.[51] As one example, Pang's group developed dual-targeted nanoparticles for highly specific prostate cancer therapy.[52] In this work, they prepared paclitaxel (PTX) loaded superparamagnetic nanoparticles (SMNPs) and then conjugated with the anti-PSMA antibodies (APSMAs) by a PEG linker (termed as PTX-HMNC-EPEG-APSMA). As described in **Figure 3a**, these nanoparticles can be actively guided to tumor tissue by an external magnet. Then, the nanoparticles are capable of entering prostate tumor cells via receptor-mediated endocytosis. This dual-targeted strategy dramatically increases the local concentration of PTX in tumor and intracellular delivery to tumor cells, thus providing a much specific and highly effective inhibition of tumor growth (Figure 3b). Their work offers a bright blueprint for human prostate cancer therapy or other metastatic and malignant tumors theranosis in clinic.

2.2.2. Nucleic Acid Aptamers

Aptamers are a kind of DNA or RNA oligonucleotides which only have one single strand. With high selectivity and affinity, aptamers can be applied to specifically binding to a wide variety of biomedical molecules including drugs, proteins, small molecules, and cancer cells.[53] Compared with antibodies, aptamers have similar targeting capacity to particular molecules and cells,[54] but on the other hand, they have superior features that make them more intriguing than antibodies. Firstly, owing to the smaller size, aptamers can afford greater tissue penetration depths. Secondly, having no immunogenic reactions and high in vivo stability enables them a good potential for future clinical applications. Thirdly, similar to nucleic acids, with good

solubility in different solvents and easy synthesis, aptamers can be chemically modified to meet different needs in individual targeting platforms.[55] In 2013, Tan and his group developed an aptamers-mediated probe to modify immune cells for recognizing and killing cancer cells.[29] They designed diacyl lipid-PEG linker-DNA aptamer conjugates and anchored them on immune cells surface, thus endowing them with specific targeting ability (Figure 3c). When cancer cells are incubated with the modified immune cells, the cancer cells can be recognized and ultimately killed. To locate lipid conjugates, a red fluorescent dye (TAMRA) was conjugated to the oligonucleotides (termed as lipo-Lib-TMR). In Figure 3d, intensive red signals are only detected on the cell membrane after treatment with lipo-Lib-TMR, indicating that the successful anchor of as-prepared lipid-DNA probe on cell surface. Figure 3e and 3f show proper recognition of targeting cells by TD05 aptamers-modified cells. Assembly and aggregation upon receptor-ligand binding are observed in the TD05-treated Ramos cells, whereas no cell-aptamer-cell assembly is found after treatment with lipo-lib-TMR. The above results demonstrate that membrane-anchored aptamers can selectively trigger cellular adhesion and this strategy provides an opportunity for achieving cell-based targeted delivery and therapy.

2.2.3. Peptides

As discussed above, antibodies and aptamers are widely utilized as escort molecules for targeted delivery of nanomaterials. However, both of them possess several unfavorable drawbacks. First, mAbs are difficult to achieve large scale manufacture due to batch-to-batch variations and complicated and expensive preparation process. Moreover, utilization of targeted mAb nanomaterials is limited by large antibody size, thus leading to worse ability to penetrate tissue as well as much more difficulty in further chemical modification or conjugation to nanomaterials. Although aptamers have better properties than mAbs, the potential nuclease degradation can induce rapid blood clearance and has aroused widespread concerns. Therefore, alternative small-sized ligands with high stability, such as peptides, have attracted considerable

attention and substantial research.[56] Peptides are particularly well suited for targeting nanomaterials because of their fascinating merits consisting of low cost, low immunogenicity, high affinity to targets, easy to synthesize\handle and long-term storage.[57] Furthermore, their small size has negligible influence on the optimized physicochemical properties of nanomaterials.[58] These superior properties make peptides as excellent targeting molecules for drug delivery and biomedical imaging.

$\alpha_v\beta_3$ integrin

Integrin belongs to the membrane spanning receptors, which has a great influence on cell signaling to operate cell shape, motion and division. Meanwhile, they are also important players to mediate the association of a cell to surrounding extracellular matrix (ECM) proteins.[59] Accordingly, the $\alpha_v\beta_3$ integrin receptors have a direct effect on human metastasis and tumor angiogenesis. More interestingly, the $\alpha_v\beta_3$ integrin receptor based targeting probe could be bound to both tumor vasculature and tumor cells because $\alpha_v\beta_3$ integrin is upregulated on both of these two places in many animal models.[60] Arg-Gly-Asp (RGD) peptide having three-amino-acid sequence possessesa high avidity for selective binding to $\alpha_v\beta_3$integrin receptor. Therefore, RGD peptide and its derivatives have been extensively developed for site-specific tumor theranosis in recent years.[41] In 2013, Angelo and co-workers explored the *iso* Arg-Gly-Asp (*iso* DGR)-conjugated human serum albumin (HSA) as a new $\alpha_v\beta_3$ selective vehicle for the delivery of drugs to tumors.[30]*Iso* DGR is a derivative of Arg-Gly-Asp (RGD), which can recognize RGD-dependent integrins with different affinity and specificity.[61] Owing to the high selectivity for tumor vessels, *iso* DGR-conjugated HSA might be exploited as a novel and versatile nanomaterial for the tumor vasculature-selective cancer theranosis.[30]

Cell-Penetrating Peptides for nucleic acid pharmaceuticals (NAPs) delivery

Cell-penetrating peptides (CPPs), also known as protein transduction domains, is a typical cationic peptide with abundant lysine and arginine amino acids, providing a better ability to rapidly translocate into almost any live cells. CPPs can readily deliver a wide range of membrane-impermeable biological molecules into cells by noncovalent complex formation or chemical conjugation.[62] This eminent performance endows CPPs with high internalization efficacy as an attractive carrier for the delivery of various cargos, in particular nucleic acid pharmaceuticals (NAPs), including short oligonucleotides, plasmids, genes, and small interference RNAs (siRNA) and their analogues. Apart from the superior membrane-permeable ability, CPPs are optimal candidates for NAPs delivery owing to their low cytotoxicity and flexible structural design.[63,64]

Despite CPPs exhibit a promising potential in NPAs delivery for quickly penetrating tissues and crossing cellular membranes, they are universal transporters being lack of receptor specificity because of their positive charges, thus hindering their practical in vivo applications.[65] To address the issue, in the past few years, substantial improvements in designing and implementing CPPs-based NAPs delivery systems have been continuously made.[66-68] In 2012, Bhatia *et al.* constructed a library of tandem CPPs that condense siRNA into stable tumor penetrating nanocomplex through noncovalent interactions for receptor-specific siRNA delivery.[69,70] Through the assistance of both tumor-specific and cell-penetrating peptides, the nanocomplex may deliver siRNA deep into the tumor site with enhanced specificity. In 2013, Shen's group applied transactivator of transcription (TAT) peptides – a kind of CCPs – to approve a molecular modification strategy for suppressing nonspecific interactions of CPPs in the blood compartment whereas remotivating their functions in the targeted tumor cells to enhance their selectivity and specificity (**Figure 4a**).[68] As the primary lysine residue amines in CCPs are the main reason of their low specificity, Shen and co-workers hypothesized that if amidization of the amines in the lysine residue to succinyl amides, it might block the functions of CPPs and thus inhibit their unnecessary interactions. As

depicted in Figure 4b, ^{a}TAT is the modified form of TAT. When the ^{a}TAT-based carrier permeates into tumor tissue by the EPR effect, these succinyl amides are hydrolyzed upon acid-triggering in the tumor extracellular acidic environment (pH < 7) and then release the pristine functioning TAT for quick cellular uptake and nuclear targeting. This smart off-on active targeting vehicle will provide a better opportunity in drug delivery based on CPPs with significantly enhanced selectivity and specificity.

2.2.4. Folic acid

Folic acid belongs to the family of vitamin B that is essential elements for cell survival and proliferation.[41] The folate receptor (FR) is a 38 kDa glycosyl-phosphatidylinositol-settled glycoprotein expressed on the membrane of many cancer cells.[12] Based on the highly selective avidity for binding to FR, folate ligands are the most widely explored targets for cancer therapeutics and diagnostics.[71-73] Beyond that, folate is characterized with many additional advantages. Firstly, compared to mAbs, small molecules like folic acid are much easier to be chemically modified and industrially manufactured at a large scale, which is an obvious advantage for clinical application.[15] Secondly, folate ligands have low cost, negligible immunogenicity, trivial toxicity, and good stability in long-term blood circulation.[74] There are two common methods of incorporating folate to drug delivery systems: one is physical adsorption, and the other is chemical conjugation by linker molecules.[73] Chen's group applied the covalent coupling strategy to anchor folic acid on layered double hydroxide (LDH) nanoparticles for targeted drug delivery with enhanced selectivity and anticancer efficacy.[71] Figure 4c described the designed strategy of this targeted LDH nanomaterials which loaded with anticancer drug methotrexate (MTX) and their significantly enhanced cell cytotoxicity was shown in Figure 4d. Compared with other control groups (FA-conjugated LDH nanoparticles without MTX, free MTX), FA-conjugated MTX-loaded LDH nanoparticles performed the best in killing cancer cells, indicating that this novel nanoplatform has practical value in targeted

drug delivery with high efficacy. Similar strategy has also been used in Lee's and Lai's work to realize the targeted delivery of small organic nanoparticles and polymeric micelles to various cancer cells.[72, 73]

2.2.5. Transferrin (Tf) and lactoferrin (Lf)

The transferrins, typically 80 kDa monermeric glycoproteins containing a single polypeptide chain with rich amino acids, plays significant role in keeping the availability of free iron and preventing the precipitation of insoluble ferric hydroxide conglomerations by binding, secluding, and transporting free iron ions in body fluid environment.[75] Although the transferrin receptor (TfR) appears to be expressed in all nucleated cells because iron is the essential trace element to all cells in the body, there is upregulation of TfR in neoplastic cells because of the requirement of high level of iron for angiogenesis and cell growth.[76] Attributed to the overexpression of TfRs on malignant cells compared to normal ones, transferrin is a very promising marker for active targeting. Similar to transferrin, lactoferrin (Lf) is also an iron ions-binding glycoprotein belonging to the Tf family and assists iron uptake in mammalian cells.[77] Recently, lactoferrin receptor (LfR) was demonstrated to undergo transcytotic pathway in the brain endothelial cells and pass through the blood-brain barrier (BBB) in vitro and in vivo.[78] Hence, Lf might be exploited for the brain-targeting delivery of therapeutic agents through circumventing the epithelial and endothelial barriers. Yang's group prepared two kinds of iron oxide nanoparticles that attached with transferrin[34] and lactoferrin.[79] The orthotopic C6 glioma xenograft was clearly visualized by these two targeting nanoparticles at 48 hours after injection. In addition, fluorescence photos affirmed the specific interaction between the modified nanoparticles and their corresponding receptors on the brain endothelial cells. Gao *et al.* developed a type of PEG-coated Fe_3O_4 nanoparticles covalently modified with Lf and applied them to act as brain MRI imaging agent both in vitro and in vivo. (**Figure 5a**)[35] Representative axial T2* images of rat brains were acquired before and after injection of Fe_3O_4-

Lf and Fe_3O_4. The Lf bonded group shows obviously enhanced signal of blood vessels (the red circles in Figure 5b-b). This suggests that the Fe_3O_4-Lfnanoparticles can potentially be used as a brain-targeting delivery vehicle for cancer therapeutics and diagnosis.

2.2.6. Other receptors

Besides the above described biomarkers, many other ligands have also been identified as possible targets of nanomaterials for specific drug delivery. These receptors are overexpressed in particular tumor cells such as matrix metalloproteinases (MMPs),[80,81] polyunsaturated fatty acids (PUFAs),[36,82] and hyaluronic acid (HA).[83,84]

Matrix metalloproteinases(MMPs), a family of zinc-dependent endopeptidases that participate in tumor aggression and progression by degrading the extracellular matrix, are overexpressed in tumor environments.[85] The membrane-type (MT) MMPs are a subset of the membrane-anchored proteinases and play predominant roles in dividing cancer cells with relevance for tumor proliferation and metastasis.[86] Thus, MMPs have become attractive targets for drug delivery and molecular imaging. More interestingly, MMPs not only can be used as a target but also might be explored for stimuli-responsive drug delivery based on MMP-cleavable peptide linkage because the MMP enzyme is up-regulated in tumor tissue.[87] Huh and Haam's groups designed a MT1-MMP-targetable magnetic nanoprobe conjugated with an activatable fluorogenic peptide to precisely recognize the expression of MT-MMPs presented on invasive cancer cells and their enzymatic activity.[81] In 2014, Zhang *et al.*developed anticancer drug doxorubicin (DOX) and plasmid DNA co-loaded graphene oxide (GO)-based nanohybrid for responsive drug delivery and cancer combination therapy.[88] First, DOX was chemically conjugated to GO via a MMP2-cleavable peptide linkage. When the nanohybrid reaches the MMP2-overexpressed tumor sites, the peptide linkage cleaved and subsequently released the drug as well as recovered the red fluorescence of DOX. This tumor triggered theranostic

strategy could open a new window for highly efficacious cancer combination therapy merging the targeting and bio-responsive capability.

Plyunsaturated fatty acids (PUFAs), possessing 18, 20, and 22 carbons and 2-6 *cis* double bonds in their backbone, are ideal candidates for tumor-specific drug delivery because they can be taken up cushily by tumors from the blood.[89] In particular, docosahexaenoic acid (DHA) is the most widely employed fatty acid as a guiding molecule approved by Food and Drug Administration (FDA), which is a basic constitute of cell membranes in the brain and other capillary endotheliums.[90] DHA-paclitaxel conjugate has already been synthesized and used in active tumor targeting. The antitumor activity in mice was exceedingly increased when compared with paclitaxel without DHA modification.[82]

Hyaluronic acid (HA) is an anionic linear polysaccharide and serves a variety of functions within the extracellular matrix. Owing to their low cost, negligible toxicity, good biocompatibility and abundant expression in most malignant solid tumors, HA has attracted intensive attention for application in targeting of anticancer agents with enhanced binding and internalization.[91] In a recent work, Bissell and co-workers made use of a fluorescent HA to observe distinctive attaching patterns to HA receptors CD44 and RHAMM. This quantitative approach not only reveals the heterogeneity of breast cancers, but also may be applicable to other ligands/receptors.[92]

Apart from these described ones, many other receptors including human epidermal receptor-2 (HER-2),[26, 93] low-density lipoprotein receptors (LDLR),[94, 95] vascular cell adhesion molecule-1(VCAM-1),[96, 97] and interleukin (IL) receptors[98, 99] have also been employed in a variety of nano-drug delivery systems for an extensive variety of applications.

2.3. Two (or more) order targeting of nanomaterials for drug delivery

As reviewed above, nanomaterials conjugated with a wide range of pertinent tumor-binding markers have been utilized to actively target malignant cells via specific receptor-ligand

interaction. The surface receptors of tumor cells vary both spatially and temporally, express differently from each other in the same tumor. Unfortunately, most receptors are only overexpressed in certain cells, targeting a single surface receptor results in uncontrollable and variable targeting delivery efficiency and thus the outcome of the single-targeting approach seems to be limited. Multidrug resistance (MDR) is a major factor in the failure of many forms of cancer chemotherapy. In particular, P-glycoprotein (P-gp) is well known to be an important drug efflux pump, which usually results in drug resistance. To overcome this problem, attractively, several recent works have showed that a dual-targeting approach can be a better option for targeted delivery and MDR reversal.[100,101] In 2014, He *et al.* developed folate and CD44 receptors dual-targeting hydrophobized hyaluronic acid paclitaxel-loaded polymeric micelles for overcoming multidrug resistance and improving tumor distribution.[101] The two (or more) order targeting has also been utilized in crossing the blood-brain barrier (BBB) in vivo. Li *et al.* developed a two-order targeted strategy to vastly improve nanoparticles' localization and visualization in U87MG glioblastoma xenograft (Figure 5d).[102] In this approach, a novel type of dual-modal imaging and two-order targeted PAMAM-G5 dendrimer nanoparticles was designed. In the system, Gd^{3+}-DOTA chelators and fluorophore Cy5.5 were employed for MIR and NIR imaging, respectively. At the same time, cyclic [RGDyK] peptides were for targeting tumor vasculatures where $\alpha_v\beta_3$ integrin is over-expressed, and Angiopep-2 peptides for facilitating the delivery of the nanoparticles across BBB. As presented in Figure 5c, dendrimer nanoparticle first binds to tumor neovasculatures that are sounding tumor periphery, and then targets to the $\alpha_v\beta_3$ integrin and LRR receptor thus leading to crossing the BBB and finally entering into brain tumor cells by the second targeting. Subsequent in vivo MIR and NIR imaging of U87MG tumor (Figure 5e) indicates the feasibility of using dual-modal nanoparticles to locate and visualize brain tumor with high specificity. In the near future, we believe that the development of two (or more) order targeting nanomaterials holds great promise for highly specific drug delivery and imaging.

2.4. Target delivery to specific subcellular action sites

In this section, we mainly focused on using targeting ligands to help drugs to achieve specific delivery to disease cells. Compared with free drugs, the nanomedicines are able to more efficiently to penetrate the cell membranes and enter into the cytoplasm. However, most of the intracellular localization of nanoparticles are observed in the cytoplasm and rarely in the cell nucleus. In fact, the cell nucleus is the final targeting site because it is the cellular "heart", which contains the genetic information and where various therapeutic agents efficiently work. Typically, the aim of gene therapy is to correct disfunctional genes by delivering therapeutic genes into the cell nucleus. In addition, certain anticancer drugs such as doxorubicin, camptothecin and cisplatin need to diffuse to nucleus for taking effective function because nucleus is their targets. To further improve the efficacy of these types of drugs and help them to target specific subcellular action sites, various approaches have been developed. For example, Parang's group reported several nuclear-targeting cyclic peptides such as $[WR]_4$ and $[WR]_5$ to form a complex with the cargo for directly transporting doxorubicin to nucleus. $[WR]_4$ and $[WR]_5$ are a kind of cyclic cell-penetrating peptide with nuclear targeting and non-covalent molecular transport capabilities. [103] In another work, Shi *et al.* developed TAT peptide-conjugated monodisperse mesoporous silica nanoparticles with high loading of doxorubicin for nuclear-targeted drug delivery. TAT peptide is an efficient ligand for translocating nanomaterials into cell nucleus through the binding import receptors and subsequently entering their nucleus. [104] Both of aforementioned nuclear-targeted drug delivery systems showed a significant enhancement in anticancer activity of doxorubicin because nuclear-targeted drug delivery strategy is expected to kill cancer cells more directly and efficiently.

3. Physical approaches for intracellular delivery

Different from chemical approaches, physical platforms are mechanically interacting with the cell membranes and thus enhance membrane permeability to achieve improved intracellular delivery. In the following part, the advancement of a number of traditional physical approaches including electroporation, sonoporation, and biolistics will be summarized. Additionally, emerging techniques using nanoneedle/nanowire arrays for high-throughput intracellular delivery will be highlighted.

3.1. Electroporation

Electroporation is to use short high-voltage electric pulses to reversibly disrupt cell membranes for delivery of wide range of molecules.[105] It is one of the most commonly used approaches in the past decades. This technique was initially developed for gene transfer and later the usage was extended to a wide variety of molecules. Under short high-voltage electric pulses, pores created on the cell membranes allow molecules to diffuse into cells. Only when transmembrane potential exceeds a threshold, electroporation aided intracellular delivery can be achieved.

Transmembrane potential is described by equation:

$$\Delta V_m = f E_{ext}\, r \cos\Phi \qquad (1)$$

Where V_m is the transmembrane potential, f a form factor of extracellular field distribution, E_{ext} the applied electric field, r the cell radius and Φ the polar angle with respect to the external field.[105]

Neumann *et al.* introduced this technique in 1982 to deliver DNA into viable mouse lyoma cells by a high electric field.[106] In this study, electric impulses (8 kV/cm, 5 μs) were applied to mouse lyoma cells. After three successive electric impulses, 95 ± 3 transformants per 10^6 cells DNA were found at a dose of 1.2 μg of DNA. This study initiated a simple physical model for enhanced DNA intracellular delivery with high electric field. In 1980s, *in vitro* electroporation was widely employed for the delivery of human kappa immunoglobulin genes into mouse pre-B Lymphocytes,[107] plasmid DNA and antisense RNA into plants cells,[108, 109]

and DNA to bacterial cells[110, 111]. From 1990s, electroporation was broadly investigated for in vivo applications[112] in muscle,[113] skin,[114] liver[115] and tumors[116,117] and successful intracellular delivery was demonstrated. Currently, electroporation has been very commonly used. Compared with the platform at 20 years ago, modern electroporation possesses improved deliver efficiency, reduced operation time, and increased cell survival rate and precision. For example, Saito *et al.* reported *in vivo* high efficiency and fast transfection of nervous systems by electroporation.[118] In their study, over ten embryos were conducted within 30 minutes and the survival and transfection rates were all greater than 90%. Geng *et al.* described a novel flow-through electroporation method for gene delivery.[119] Compared with conventional electroporators working in bath mode and being limited by performing on only small amounts of samples (about 1 mL in volume) each time, this modified approach can continuously deliver gene into Chinese hamster ovary (CHO-K1) cells in a high-throughput way (up to about 20mL/min) with a high transfection efficacy of 75%. Boukany *et al.* further combined electroporation with nanotechnologies and introduced a type of nanochannel electroporation, which can deliver precise amount of biomolecules into living cells with minimal cell damage.[120] This device includes two microchannels (cell in one side and reagent in the other side) that are linked with a nanochannel. When high voltage pulses are applied between the two microchannels, a limited area of cell membrane is affected by intense electric field, thus allowing very precise amount of biomolecules into cytoplasm. Overall, electroporation offers many great advantages including 1) high efficiency for gene and other biomolecules intracellular delivery, 2) minimal safety concerns, 3) cell type independency. However, electroporation requires very complicated pulse generators to produce short high-voltage electric pulse and expertise is needed for equipment handling.

3.2. Sonoporation

Sonoporation is an approach to facilitate intracellular delivery by applying ultrasound. High-amplitude acoustic wave induced cavitation is considered as the main mechanism of sonoporation for intracellular delivery. Stable cavitation and transient cavitation are two typical cavitation forming modes.[121] The former is the stable oscillation of encapsulated gas bubbles, inducing shear forces to disrupt cell membrane and leading to allow drug/gene delivery. The latter is caused by collapse of bubbles which generate shock waves to facilitate intracellular delivery. Sonoporation is a reversible and non-destructive process. Cells can recover their membranes within a few seconds after ultrasound exposure.[122] Although ultrasound has been used for diagnosis for many years, its applications for gene delivery just started in mid-1990s. Kim *et al.* and Bao *et al.* reported successful *in vitro* transfection of plasmid DNA with help of ultrasound.[123, 124] Later, ultrasound was used for *in vivo* naked DNA delivery. For example, skeletal muscle,[125] carotid artery,[126] kidney,[127,128] and heart[129] were tested with successful DNA transfection using ultrasound.[130] Besides gene delivery, ultrasound was also used for transdermal drug delivery. For example, ultrasound can increase the permeability of skin by disrupting stratum corneum lipid structure, thus facilitate drug/biomolecules penetrate into skin tissue.[131] Generally speaking, there are two kinds of approaches for local drug delivery using sonoporation.[131] One is to use micro- and nanobubbles to help to create cavitation on the cell membranes and disrupt blood vessels, thus increasing permeability of certain cells and facilitating drug localization in tumors. The other is to directly load drug into microbubbles. In this approach, ultrasound induced microbubbles collapse to not only create cavitation, but also trigger release of drug molecules to ultrasound treated cells. Ultrasound is shown to be promising for cancer tumor treatment. Drug/gene encapsulated carriers can be injected into mouse and pulsed focused ultrasound is subsequently applied to tumor tissues for triggering drug release or improving drug delivery by destructing micro- and nanobubbles.[132] For example, Lin *et al.* reported enhanced tumor deposition of lipid-coated CdSe quantum dot with aid of ultrasound and an ultrasound contrast agent.[133] In this study, an ultrasound contrast agent

(microbubbles) was intravenously injected followed by ultrasound application to tumors. Then lipid-coated CdSe quantum dots were injected. The results demonstrate that the application of ultrasound and microbubbles is able to enhance drug delivery into the tumors possibly by increasing vascular permeability.[133] Suziki *et al.* reported a type of liposomal nanobubbles with size of 500 nm for dramatically enhanced plasmid DNA (interleukin-12 corded plasmid DNA) delivery and correspondingly an effective gene therapy for cancer treatment.[134] Other than using micro- and nanobubbles purely as a cavitation generation medium, Yang *et al.* presented using Fe_3O_4 nanoparticles loaded microbubbles for controlled release to tumor cells through sonoporation. Through this approach, the nanoparticles can be effectively delivered into tumor cells with a noninvasively way through help of ultrasound, and the delivery rate can be controlled by acoustic intensity.[135] Moreover, focused ultrasound is able to successfully create blood-brain barrier disruption (BBBD) without neuron damages,[136] through which chemotherapy agent or antibody, like Doxorubicin and Herceptin, can nan-invasively transport into rat brain with increased local concentration.[137,138] Most recently, Ting *et al.* fabricated anticancer drug 1,3-bis(2-chloroethyl)-1- nitrosourea (BCNU) loaded microbubbles and found that the nanomedicine improves local BCNU deposition in brain tissue under focused ultrasound. In vivo study shows the BCNU loaded microbubbles can effectively inhibit tumor growth (tumor size of 11.0 ±1.0 mm^3 in comparison with control group of202±24.10 mm^3).[139]

3.3. Biolistics

Biolistics (also called as gene gun) is a method to inject cells with very high speed heavy metal particles coated with DNA for gene delivery.[122] This technique was introduced by Klein *et al.* in 1987 for gene transfection of plant cells.[140] In the study, accelerated small tungsten particles carrying RNA/DNA pierced cell walls and membranes and penetrated into intact plant cells without noticeable cell death.[140] In early 1990s, this approach was extended to mammalian cells and also liver and skin tissue in living mice.[141, 142] The studies proved that biolistics is

effective with a wide range of isolated cells and tissues. O'Brien *et al.* used a hand-held gene gun and realized successful transfection of cultured human embryonic kidney (HEK) 293 cells and organotypic brain slices.[143] Although many studies reported biolistics method can deliver gene and transfect cells in many organs (e.g., muscle, liver, heart and brain), the major application is transdermal delivery because its low tissue penetration capacity.[130] Rakhmilevich *et al.* reported gene gun-mediated skin transfection of interleukin 12 can effectively inhibit tumor growth.[144] In their study, detectable level (266.0 ± 27.8 pg) of transgenic protein was found at treated skin sites. This method was able to completely regress the established tumors in the treated mice. In biolistic delivery, particles can penetrate the outer layer of skin and reach the epidermal layer rich of antigen presenting cells, thus maximizing immunization efficiency. Larregina *et al.* reported transfection of human skin organ cultures with antigen expression in resident cutaneous dendritic cells by biolistics.[145] They also found the particles are primarily in epidermis, and Langerhans cells can be successfully transfected. Hung *et al.* tested delivery of several DNA vaccines through biolistics method for cervical cancer vaccination.[146] Beyond heavy metal nanoparticles which may cause adverse side effects, a number of types of biodegradable polymeric nanoparticles were also chosen for biolistic applications. Lee *et al.* developed a biodegradable and non-toxic polymeric nanoparticles composing of chitosan and poly-γ-glutamic acid for transdermal DNA delivery with help of low pressure gene gun.[147] The results indicated good penetration depth was obtained in mouse skin and *in vivo* gene expression was realized. Despite of the great potential of biolistics for transdermal gene delivery, this technology is seriously limited by several shortcomings: 1) high-cost of equipment, 2) high cell damage and death, 3) variation of skin properties.[148]

3.4. Nanoneedles/nanowires

Microinjection was broadly used for delivering exogenous molecules into cells by penetrating the membranes with minimal perturbation. In 2007, it has be found a single multiwalled carbon

nanotube attached to an atomic force microscope (AFM) tip can deliver protein coated quantum dots into living human cells with a nanoscale resolution.[149] This nanotube causes no discernible membrane or cell damage, and can deliver a discrete number of molecules to the cell's interior without the requirement of a carrier solvent.[149] Two years later, Yum *et al.* used boron nitride nanotube to deliver fluorescent quantum dots into living cells (**Figure 6a**, b).[150] In this study, they demonstrated the selective delivery of monodispersed quantum dots into the cytoplasm (Figure 6c) or nucleus (Figure 6d) of living cells.[150] After that, Singhal *et al.* reported carbon-nanotube based endoscopes for minimally invasive intracellular probing, drug delivery and single-cell surgery (Figure 6e).[151] This endoscope can very precisely enter specific area of cells with about 100 nm resolution and also access organelles without disrupting the cells (Figure 6f). Also this endoscope is able to transport nanoparticles and attoliter volumes of fluids to and from precise locations with a minimal invasive way.[151] Although these approaches can effectively deliver molecules/particles with ultrahigh precision, the technique can treat only very low number of cells at one time and not applicable for mass intracellular delivery of drugs/molecules. This drawback seriously limits this technique for future clinical applications.

To achieve high-throughput intracellular delivery, Kim *et al.* first demonstrated applying a silicon nanowire (SiNW) array for this purpose. In the technique, mammalian cells such as mouse embryonic stem (mES) cells and human embryonic kidney (HEK 293T) cells were cultured on the top of SiNWs pre-coated with DNA (**Figure 7a-b**).[152] Through the study, they observed that the cells could be pierced by the SiNWs and gene transfection of HEK 293T cell line was achieved. However, a low transfection efficiency, less than 1%, was obtained. Shalek *et al.* later demonstrated SiNWs can penetrate the cell membranes and subsequently release the surface-bound molecules directly into cell's cytosol.[153] It was also found that this modality can deliver a wide range of molecules for versatile applications including delivery of small molecules for guided neuronal progenitor growth, siRNAs for gene silence, and peptide for

inhibiting apoptosis. With this method, a large number of cells can be cultured on SiNWs at the same time. Therefore this platform can introduce a diverse range of biomolecules to living cells in high-throughput way without need of chemical modification and viral packaging. Recently, Melosh and co-workers developed alumina hollow nanostraw arrays(Figure 7c).[154] The nanostraws (nanotubes) can penetrate the cell membranes during cell culture on top of them. Then protein/genetic molecules can be injected into the cells through an integrated device, as shown in Figure 7d. Later on, the same group equipped the nanostraw array together with electroporation platform. With help of electroporation, highly efficient molecule delivery and high transfection yields with excellent uniformity and cell viability were achieved.[155] In the two studies, they found hollow nanostraw platform can offer high efficiency and excellent spatial, temporal and dose control for biomolecules delivery. Chan *et al.* reported vertical silicon nanoneedle arrays to transfer intact 3D DNA nanocages directly to cytoplasm without endocytosis.[156] Same as others, they also found this approach offers high gene delivery efficiency and low cell toxicity. Most recently, Melosh and co-workers demonstrated the hollow nanowires (or nanostraw) can directly penetrate membrane by observing dynamic ion delivery. They found that 7.1±2.7% of the overall nanostraws penetrated into cells and 10.7±5.8 nanostraws pierced each individual cell.[157] Generally speaking, nanowires are commonly considered as a useful tool for interfacing living cells with minimal cellular perturbation. However, culturing cells on silicon nanowire arrays might have influences. For example, mesenchymal stem cells cultured on silicon nanowire show significantly different behavior of adhesion, proliferation and differentiation, comparing with flat silicon or control substrates. This interaction between mesenchymal stem cells and SiNWs can induce the stem cells differentiating toward osteocytes and chondrocytes instead of adipocytes in the absence of supplementary growth factors.[158] In addition, Persson *et al.* reported fibroblasts cultured on nanowires exhibiting low motility, impaired cell division and DNA damage.[159] They found highly curved but intact nuclear membranes, indicating no direct penetration into nuclei. DNA

602 damage may be induced by reactive oxygen species (ROSs) which were triggered by cellular
603 stress and high respiration rates.

604 Different from culturing cells on nanowire or nanostraw arrays, we designed a novel
605 approach using nanoneedle arrays to actively and mechanically disrupt cell membrane for
606 intracellular delivery.[160] The principle of this approach is similar with employing microneedle
607 arrays to pierce the stratum corneum for transdermal drug delivery. Transdermal delivery is
608 using microneedle to penetrate stratum corneum to facilitate vaccine/drug diffusion.[161,162] To
609 avoid irreversible damage to cells but maintain high intracellular delivery efficiency, we
610 decided to fabricate nanoneedles with diameter of 100-400 nm. To ensure the nanoneedles have
611 enough mechanical strength to pierce the cell membranes when the diameters are so small, we
612 selected diamond, the hardest material in nature, to make robust nanoneedle arrays.[163] The
613 diamond nanoneedle array was fabricated by depositing a layer of diamond film on a silicon
614 substrate followed by bias-assisted reactive ion etching (RIE). The prepared diamond
615 nanoneedles have distal tip diameter of 135 ± 20 nmand length of 7.42 ± 1.35 μm (**Figure 8a**).
616 Different from others' culturing of cells on nanowires or nanostraws for passive penetration
617 into cells for intracellular delivery, we applied cell suspension onto the diamond nanoneedles
618 for active disrupting the cell membranes within extremely short period of interaction time.
619 Figure 8c-e clearly demonstrates that dramatically improved intracellular delivery of
620 luminescent iridium (III) polypyridine complex when the cells were treated with nanoneedles.
621 Very attractively, direct nucleus delivery of molecules was confirmed to be achievable. Beyond
622 facilitating delivery of fluorescent molecules to cells, nanoneedle arrays can also greatly
623 enhance the delivery of biologically functional molecules to cells and increase their therapeutic
624 efficacy (Figure 8f). With similar strategy, high density diamond nanocone arrays (200 nm to
625 1 μm of height and 10 nm of tip radii) were shown to facilitate intracellular delivery of
626 differentiation medium to a great number of MC-3T3 cells and therefore speed up the
627 proliferation of the cells, which is potentially useful for early bone formation.[164] Besides

intracellular delivery, there are many other applications of nanowire/nanoneedle arrays. For example, Robinson *et al.* reported vertical nanowire electrode arrays can allow interfacing multiple mammalian neurons, intracellularly record and stimulate neuronal activity, and map multiple individual synaptic connections.[165] Xie *et al.* presented intracellular and extracellular long time recording of action potential of cardiomyocyte, with high signal strength and quality, using a similar approach.[166] Since 2007, majority of researches has been mainly working on *in vitro* applications of nanoneedle/nanowires. In early 2014, Tseng and co-workers reported *in vivo* study of substrate-mediated gene delivery, as shown in **Figure 9**.[167] In their deliver system, two nanoscale features were included: 1). DNA-SNPs (supramolecular nanoparticles) vector for gene encapsulation and 2). Adamantane (Ad)-grafted silicon nanowire substrates. In this research, Adamantane (Ad)-grafted silicon nanowire substrates was subcutabeously transplanted into one side of mouse body (opposite side without Adamantane (Ad)-grafted silicon nanowire substrates was considered as control) and gene encapsulated SNP was locally injected, as shown in Figure 9a. It is found that significantly increased gene expression can be achieved in presence of Adamantane (Ad)-grafted silicon nanowire substrates. This study suggests the promising potential of nanowire substrates for *in vivo* application of intracellular gene/drug delivery.

3.5. Physical stimuli aided intracellular delivery and drug release

Besides using electroporation, sonoporation, micro/nano-injection, and nanoneedle arrays for intracellular delivery, other physical strategies are also available for this purpose by stimuli-triggered delivery. The stimuli can be divided into two parts: internal stimuli and external stimuli for triggering release. For internal stimuli, local differences of pH, temperature and redox microenvironment are major potential stimuli trigger factors.[168] For instance, pH values of solid tumors are much lower (about pH=6.5) than those in blood or normal tissues (pH=7.4). pH sensitive molecules, like pH (low) insertion peptide (pHLIP), can be used to target acidic

tumor tissues.[169] Also, further lower pH values can be found in endosome and lysosome during endocytosis. Such a pH difference is often used for designing pH sensitive vesicles for increasing intracellular delivery or triggering release in tumor area.[170] In addition, the pH buffering effect of pH sensitive polymers can help endosome escape by inducing osmotic swelling and endosome rupture.[171] For external stimuli, magnetic, ultrasound, light and heat can as well be used for improved intracellular delivery or as a release trigger. For example, magnetic nanoparticles can carry therapeutic molecules into cells more efficiently and, attractively, deliver to specific area of interest with the aid of an external magnetic field.[172, 173] Light responsive nanocarriers can be triggered by near-infrared (NIR) light illumination for drug release. For example, NIR-responsive mesoporous silica coated upconverting nanoparticle can release drug by 980 nm light irradiation.[174] Extensive discussion of stimuli-responsive delivery is beyond the scope of this review, several excellent reviews are available in literature for further description.[168, 175]

4. Conclusion and future outlook

The emergence of micro- and nanotechnologies opens up new opportunities for intracellular delivery. In this review, we summarized different strategies including chemical and physical platforms to achieve improved intracellular delivery. In chemical approaches, a broad range of targeting ligands (antibodies, aptamers, peptides, small molecules, etc.), which have strong affinity with the receptors overexpressed on particular tumor cells, are modified onto the surface of nanomaterials for active targeted delivery with high specificity and selectivity. Physical approaches, including traditional electroporation, sonoporation, and emerging techniques using nanoneedle/nanowire arrays, can disrupt the cell membranes to enhance its permeability and facilitate intracellular delivery. These techniques can be tailored for high precision single cell delivery with resolution within 100 nm or for high-throughput intracellular delivery to millions

of cells. Substantial progress has already been made, but there is still an urgent need to develop one individual platform to combine as many as the advantages of different techniques such as being efficacious, specific, controllable, safe, universal and high-throughput. For clinical applications, targeting ligands will be the most promising approaches for specific delivery of drugs to certain types of cells (e.g., cancer cells), as this will lead to tremendous advantages including improved drug efficacy and reduced side effects. This approach has been used in clinical applications. For example, many mAb-drug conjugates have advanced to human clinical trials. A mAb conjugated calcheamicin (Mylotarg) has been approved for clinical use.[176] In adddition, the DHA conjugated paclitaxel (Taxoprexin) exhibited substantially increased antitumor activity, reduced systemic toxicity and high stability in blood plasma as compared to paclitaxel, which has advanced to human phase III clinical trials.[37] Despite of the great potential, there are also major challenges in systemic administrations of these targeted nanomedicines. For instance, the toxicity and the clearance of nanomedicines need to be thoroughly investigated. After taking their functions, nanomaterials are required to be excreted out from patients relatively quickly. In order to achieve this, one of the strategies is to allow nanomaterials to decompose immediately after releasing the loaded drugs to specific disease sites.[177] Another approach is to reduce unnecessary use of drug carriers. For this, carrier-free drugs are developed. [178, 179] In comparison, the main physical approaches (e.g., electroporation and nanoneedle) summarized in this review are predominantly for in-vitro studies although they can also be indirectly used for in-vivo applications. As one example, these physical techniques may be used to introduce genes to stem cells for subsequent cell therapy for patients. Overall, we believe that micro- and nanotechnologies will draw a bright blueprint for in vitro and in vivo drug delivery and further for actual clinical application.

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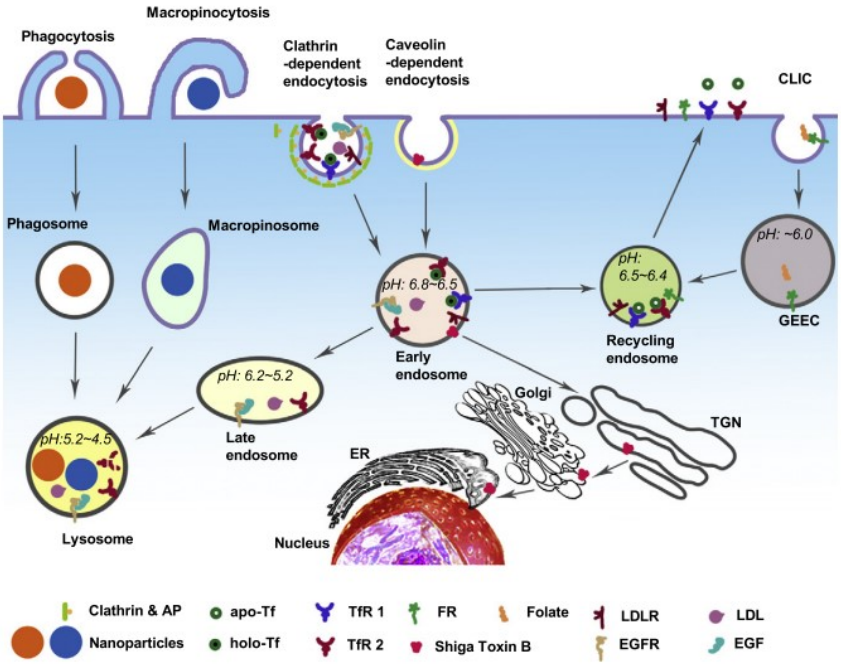

968

969 **Figure 1**. Distinct pathways for cargo internalization by cells. 1) Phagocytosis involves surface
970 receptors and engulfs large particles through envelopment by the plasma membrane. 2)
971 Macropinocytosis generates large macropinosomes containing extracellular fluid and soluble
972 protein. 3) Clathrin-dependent endocytosis involves the assembly of clathrin and adaptor
973 proteins on a region of the plasma membrane in which particular receptors are clustered to form
974 a nascent vesicle destined for internalization. 4) Caveolin-dependent endocytosis involves the
975 assembly of caveolin coats on regions of the plasmamembrane rich in particular lipid rafts to
976 form a nascent vesicle destined for internalization. Reproduced with permission.[15] Copyright
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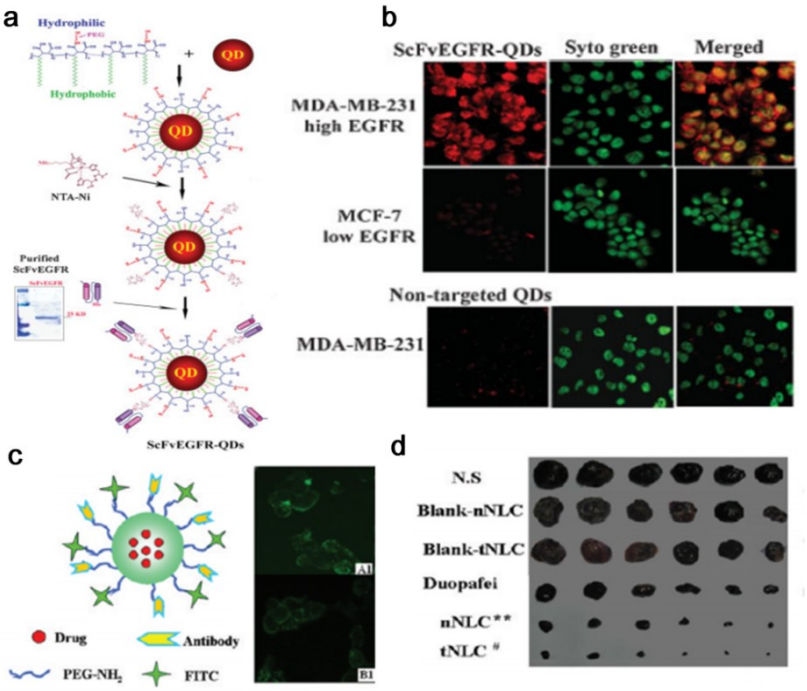
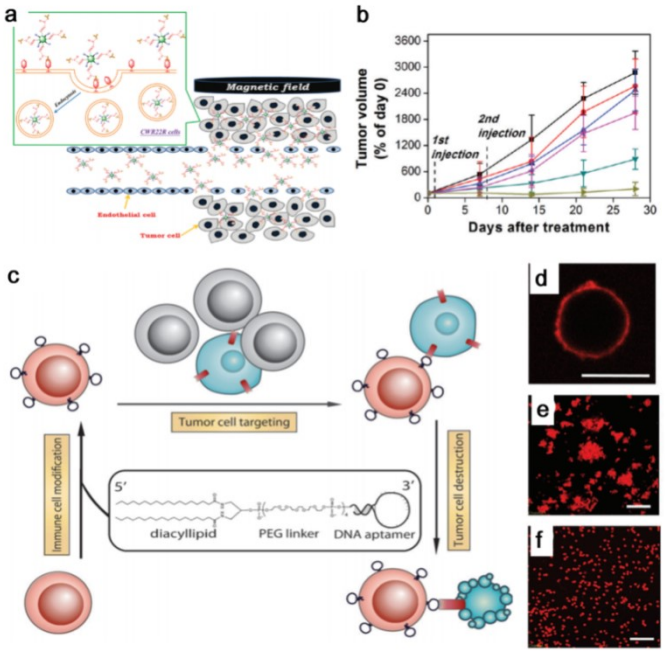


Figure 2.a) QDs were coated with the amphiphilic polymer modified with short PEG chains and conjugated to Ni-NTA. Recombinant ScFvEGFR protein has a high purity showing as a single band protein with a molecular weight around 25 kDa. His-tagged ScFvEGFR was conjugated to QDs through the interaction of nickel with histidine residues located at the C-terminal of the protein. b) Selective internalization of ScFvEGFR-QDs in tumor cells was determined using cancer cell lines expressing a high (MDA-MB-231) or low (MCF-7) level of EGFR. Strong red fluorescent signal was detected inside MDA-MB-231 cells incubated with ScFvEGFR-QDs but not with non-targeted QDs. A very low level of red fluorescence was seen in MCF-7 cells after incubating with ScFvEGFR-QDs. Cell nuclei were counterstained with Sytogreen (Invitrogen). Reproduced with permission.[45] Copyright 2009, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. c) Schematic representation of the antibody, FITC-colabeled nanostructured lipid carriers and fluorescence microscopy images of (A) tNLC and (B) nNLC.d) Photographs of tumors from each treatment group excised on day 20. Reproduced with permission.[48] Copyright 2011, American Chemical Society



995

996 **Figure 3.**a) The mechanism of action of PTX-HMNC-EPEG-APSMA for targeted cancer
997 chemotherapy in a magnetic field. b) Quantitative analysis of the effects of various treatments
998 on tumor size. Values are means (SD (n = 8). Reproduced with permission.[52] Copyright 2012,
999 American Chemical Society. c) Illustration of targeting cancer cells (blue) with aptamer-
1000 modified immune cells (red). After incubating with lipo-aptamer probes (shown in expansion),
1001 immune cells recognize and kill cancer cells in the cell mixture. d) Confocal microscope image
1002 of lipo-Lib-TMR-treated CEM cells. Red fluorescent probes were found only on the cell surface.
1003 Scale bar: 10 mm. e) Ramos cells spontaneously aggregate after treatment with lipo-TD05-
1004 TMR. Scale bar: 100 mm. f) Control experiments showed no assembly when Ramos cells were
1005 treated with lipo-lib-TMR. Scale bar: 100 mm. Reproduced with permission.[29] Copyright 2013,
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1007

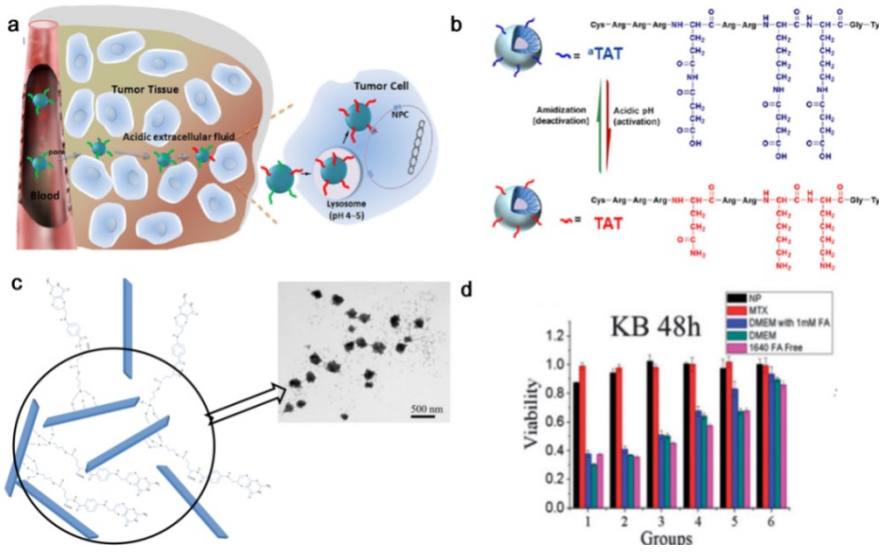
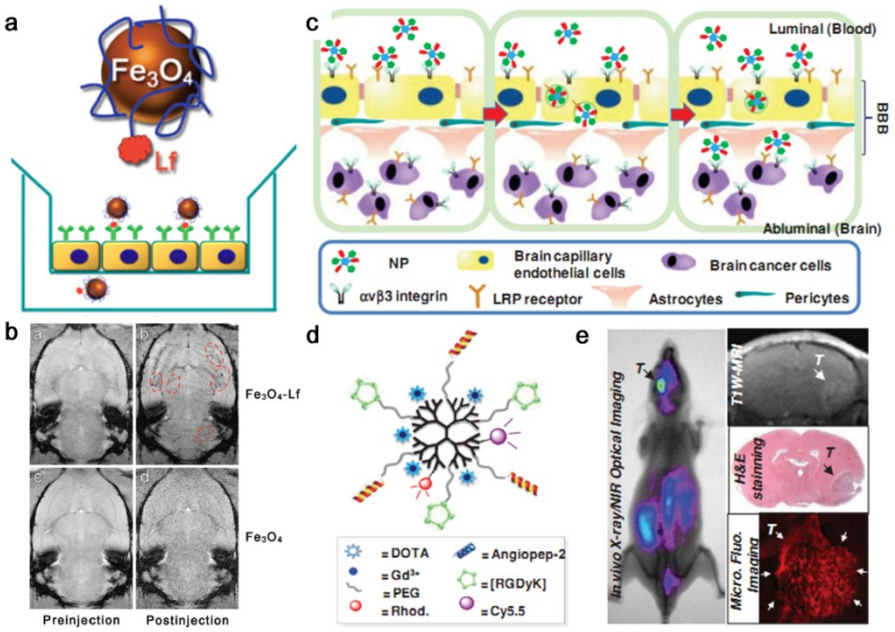


Figure 4.a) Illustration of the use of TAT as an example of a cell-penetrating peptide (CPP) to demonstrate the concept of deactivation of a CPP in the blood compartment and its activation in the tumor interstitium or cells for in vivo tumor-targeted drug delivery. The amines of the lysine residues of a CPP are amidized to inhibit its nonspecific interactions in the blood compartment without affecting the nanocarriers' stealth properties. Once the nanocarrier extravasates into tumor tissue through highly permeable blood vessels via the EPR effect, these amides are hydrolyzed, regenerating the pristine functioning CPP in the acidic tumor extracellular fluids (pH < 7) for fast cellular uptake or in acidic endo/lysosomes for fast endo/lysosomal escape and nuclear targeting. b) Amidization of TAT's primary amines to succinyl amides and their acid-triggered hydrolysis. Reproduced with permission.[68] Copyright 2012, American Chemical Society. c) Overview of FA-conjugated self-assembled LDH amorphous nanoparticles. Black arrow shows the TEM image of monodispersed FA-conjugated LDH nanoparticles. d) The viabilities of HeLa cells after 48 hours of incubation with FA-conjugated LDH nanoparticles (NP), free MTX anticancer drug (MTX) and the LDH nanoparticles loaded with MTX (NP–MTX). Reproduced with permission.[71] Copyright 2013, Royal Society of Chemistry.



1026

1027 **Figure 5.**a) Receptor-mediated delivery of PEG coated Fe_3O_4 nanoparticles were covalently
1028 bonded with Lf across the Blood-Brain Barrier. b) Axial T2* images of rat brains captured
1029 preinjection and 15 min postinjection of Fe_3O_4-Lf and Fe_3O_4, respectively. The red dashed-line
1030 circles highlight the brain blood vessels enhanced by the Fe_3O_4-Lf probe. Reproduced with
1031 permission.[35] Copyright 2012, American Chemical Society. c) Overview of two-order targeted
1032 brain tumor imaging strategy. The nanoprobe first targets the $\alpha_v\beta_3$ integrin on tumor
1033 vasculatures. After binding with nearby LRP receptors, the nanoprobe traverses BBB via LRP
1034 receptor-mediated transcytosis and finally targets tumor cells directly. d) Schematics of the
1035 targeted nanoprobes. e) In vivo MIR image and NIR image of U87MG tumor indicated the
1036 feasibility of dual-model nanoparticles to visualize brain tumor with high specificity.
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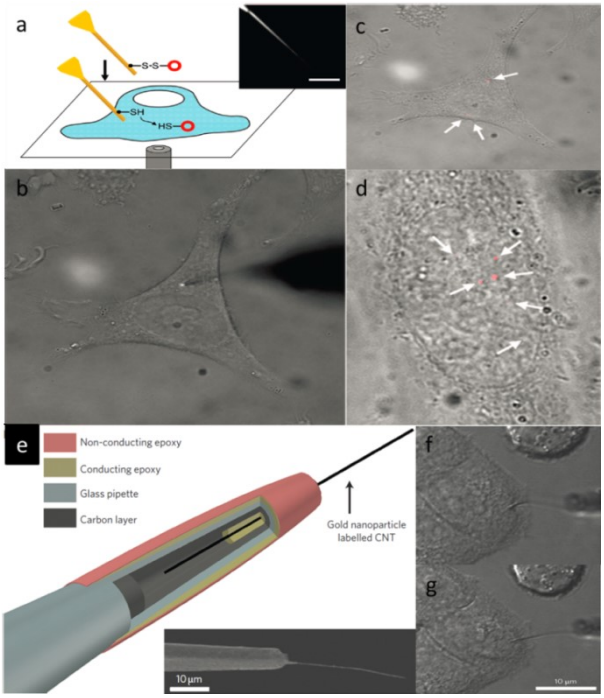

Figure 6. Nanoscale mechanochemical delivery of QDs into living cells. (a) Schematic of the mechanochemical delivery of QDs into living cells. Inset, optical microscopy image of a typical nanoneedle. Scale bar, 5 μm.(b) Optical microscope images of a nanoneedle functionalized with QDs during the QD delivery experiment, showing the nanoneedle penetrating through the cell membrane. (c-d) Overlay of bright-field and fluorescence image of the cell after the QD delivery into c) cytoplasm and d) nuclear. Reproduced with permission.[150] Copyright 2009, American Chemical Society. (e) Schematic of the nanotube endoscope. A multiwalled carbon nanotube is attached to the end of a glass pipette, which is coated with a non-conducting epoxy on the outside and a conducting epoxy on the inside. Inset: Scanning electron micrograph of as-assembled endoscopes with 100 nm carbon nanotube tips. (f-g) 100 nm nanotube tip of the endoscope f) bending and g) elastically recovering its shape when pushed against a cell membrane. Reproduced with permission.[151] Copyright 2010, Nature Pulishing Group.

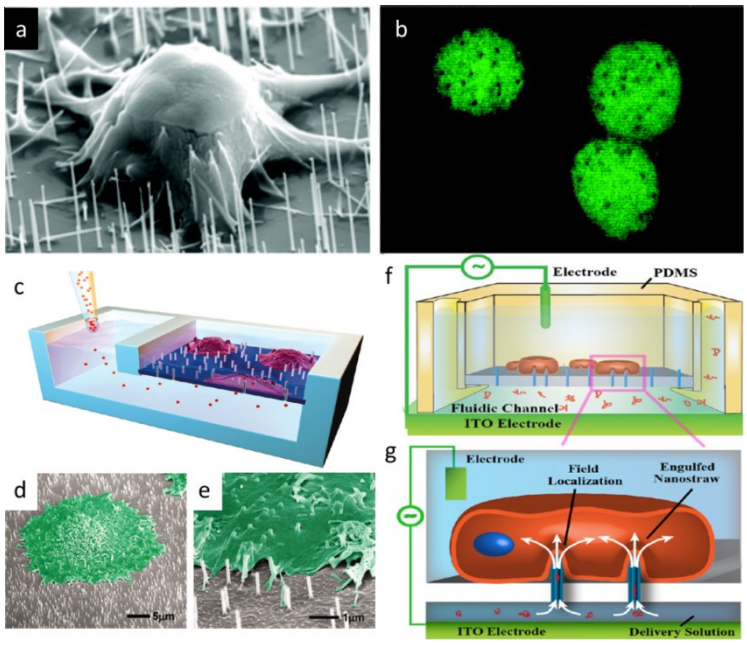

Figure 7. (a) SEM images of individual mouse embryonic stem (mES) cells penetrated with silicon nanowires. The diameter and the length of the nanowires are 90 nm. (b) A confocal microscopy image of mES cells penetrated with silicon nanowires. Reproduced with permission.[152] Copyright 2008, American Chemical Society. (c-e) Device schematic overview. (c) A cross section of a typical device used to deliver biomolecules into cells via nanostraw-mediated delivery. (d, e) SEM images of critical point dried (CPD) cells cultured on nanostraw membranes (false colored green) with 100 nm diameter straws at a density of 108 straws/cm^2. Reproduced with permission.[154] Copyright 2012, American Chemical Society. (f, g) Illustrations of nanostraw-electroporation system. (f) Schematic view of device. (b) Schematic illustration of field localization and biomolecule confinement at the tip of the nanostraw due to close contact at the nanostraw- plasma membrane interface. Reproduced with permission.[155] Copyright 2013, American Chemical Society.

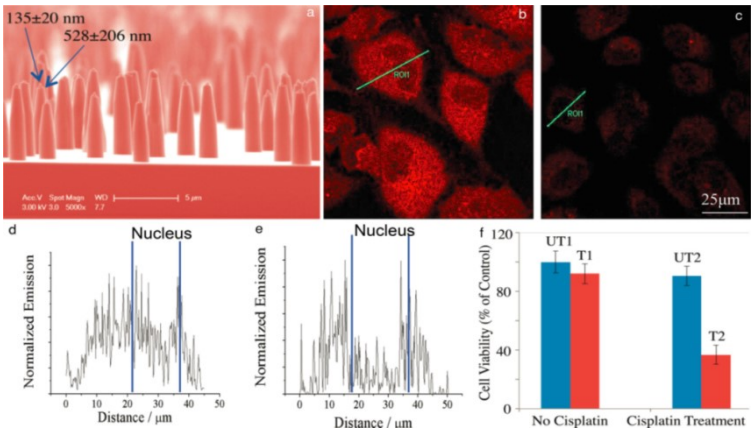

Figure 8. (a) SEM image of diamond nanoneedles; (b,c) confocal microcopy images of b) diamond nanoneedles-treated cells and c) untreated cells c), after 19 hours of incubation with luminescent iridium (III) polypyridine complex; (d) and (e) show the normalized emission intensity across the lines drawn over the cells in (b) and (c), respectively; The scale bars in (b) and (c) are 25 µm. (f) The viability of cells 72 h post plating. The cells were treated with diamond nanoneedles, cisplatin or none or both. UT (shown in blue) and T (shown in red) indicate that the cells were untreated or treated with nanoneedles, respectively. UT1: the cells were treated by neither nanoneedles nor cisplatin; T1: the cells were treated with nanoneedles but not cisplatin; UT2: the cells were treated by cisplatin but not nanoneedles; T2: the cells were treated by both nanoneedles and cisplatin. Reproduced with permission.[163] Copyright 2013, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

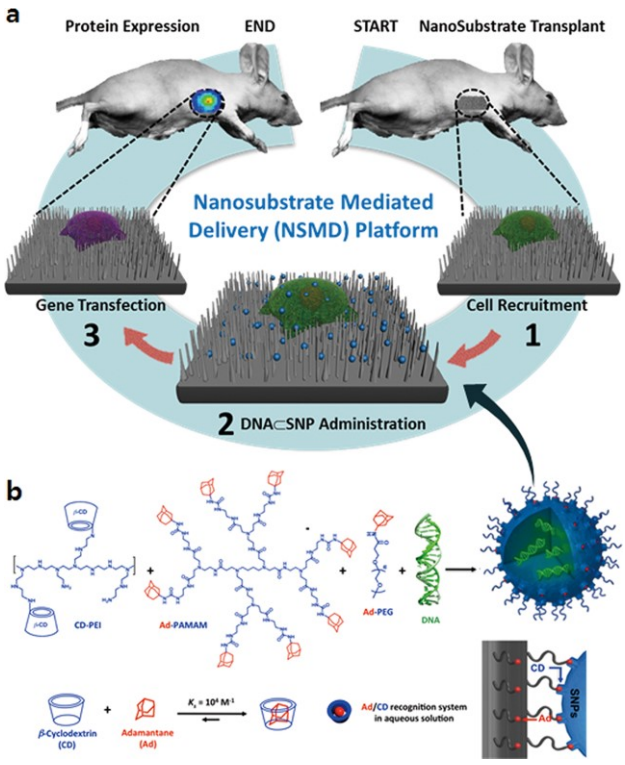

Figure 9. (a) Schematic illustration of the unique mechanism governing the nanosubstrate-mediated delivery (NSMD) approach for both in vivo and in vitro settings. (b) The multivalent molecular recognition between the Ad motifs on Ad-SiNWS and the β-cyclodextrin (CD) motifs on the surfaces of SNPs leads to dynamic assembly and local enrichment of SNPs onto Ad-SiNWS. The Ad/CD recognition system is also responsible for the supramolecular assembly of DNA⊂SNPs from the three molecular building blocks (i.e., CD-PEI, Ad-PAMAM, and Ad-PEG) and plasmid DNA. Reproduced with permission.[167] Copyright 2014, American Chemical Society.

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The Cell membranes, a biological barrier, strictly limit intracellular deliver of drugs and biomolecules, resulting poor therapeutic efficiency. In this review, we will focus on summarizing the nanomaterial routes involving in making use of a collection of receptors for targeted delivery of drugs and biomolecules and the physical ways of applying micro- and nanotechnologies for high-throughput intracellular delivery.

Keyword:intracellular delivery;targeted delivery;cell receptors;nanoneedle arrays

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Micro- and nanotechnologies for intracellular delivery